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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Gurney et al.	)	For: Alzheimer's Disease Secretase, APP Substrates Therefor, and Uses Thereof
Examiner: S. Turner	)	
	)	
Group: 1647	)	

DECLARATION OF MICHAEL BIENKOWSKI, Ph.D. PURSUANT TO 37 C.F.R. § 1.132

Commissioner for Patents
Washington, DC 20231

Sir:

I, Michael Jerome Bienkowski, Ph.D., hereby declare as follows:

I. Introduction

1. I am a co-inventor of Asp2 subject matter claimed in various patent applications filed by Pharmacia & Upjohn. I make this declaration to provide information to the Patent Office that may be relevant to patent issues relating to enzymatically active, "transmembrane-deleted" forms (ΔTM) of the Asp2 protein and polynucleotides which encode such protein. When I refer to "I" or "we" in this declaration, I mean me and/or my co-inventors and/or people working under our direction at Pharmacia & Upjohn.

2. The term "Asp2" is the name that we gave to aspartyl protease polynucleotides and polypeptides that we isolated and described in the patent applications. At least two human and one murine form of Asp2 are taught in the patent applications. Through experiments described in the patent applications we demonstrated that Asp2 exhibits proteolytic activity towards amyloid precursor protein (APP) involved in processing APP into amyloid beta (Aβ), a peptide implicated in Alzheimer's Disease pathology.

II. Cloning of Asp2 and Identifying the Asp2 transmembrane domain

3. My co-inventors and I performed and/or directed experiments which resulted in the identification and cloning of human Asp2 cDNAs. Our earliest experiments did not immediately yield full-length Asp2 cDNAs. We first obtained and sequenced two

partial clones denoted as clone 4386993 (hereinafter '438) and clone 2696295 (hereinafter '269). As explained in our patent applications, Clone '438 contains additional codons sequence at its 5' end relative to clone '269, but Clone '269 contains 25 additional codons (75 basepairs) as an internal insertion relative to Clone '438. (These 25 codons represent the difference between the long and short forms of full length human Asp2 in Figures 2 and 3 of the patent applications.)

4. After we sequenced the '438 and '269 clones we aligned the sequences with sequences of other aspartyl proteases as part of our analysis of them. From these alignments and other analysis we deduced that these sequences were incomplete cDNA sequences that were truncated at the 5' end (the amino-terminus of the encoded polypeptide). Computer-aided analysis of the predicted amino acid sequences indicated that the predicted amino acid sequence encoded by both '438 and '269 contained the DTG/DSG sequences indicative of the aspartyl protease active site, and were complete to the carboxyl-terminus of the encoded polypeptide.

5. By analyzing the partial Asp2 sequence from the '438 and '269 clones described in paragraph 3, we deduced that Asp2 contained a transmembrane domain. Our U.S. Provisional Application No. 60/101,594, filed September 24, 1998, describes the analysis as follows:

> Routine computer-aided analysis of the predicted amino acid sequence of Hu-Asp2a and Hu-Asp2(b) for secondary structure motifs resulted in detection of a predicted transmembrane domain in each polypeptide, which corresponds to Hu-Asp2(a) amino acid residues 367-392 of SEQ ID NO: 4, and of the sequence given in Figure 2, and to Hu-Asp 2(b) amino acid residues 392-417 of SEQ ID NO: 6, and of the sequence given in Figure 3.
>
> (See U.S. Provisional Application No.60/101,594 at p. 20.)

As I explain in greater detail below, the stated location of the transmembrane sequences (367-392) and (392-417), through an inadvertent error, do not correspond to the transmembrane regions of the full length human Asp2(a) and Asp2(b) proteins shown in the Figures, and standing alone, these numbers would not serve as a basis for identifying the transmembrane region of the human Asp2 sequences. However, our routine computer-aided

analysis did, in fact, permit us to identify the Asp2 transmembrane region, and a molecular biologist of ordinary ability who read the application and (through the guidance of the application) performed his/her own routine computer-aided analysis would have identified the correct location of the transmembrane region in our Asp2 sequences.

6. Through our continued research we ultimately cloned additional 5' (amino terminal) cDNA sequence for the two human Asp2 enzyme isoforms. As reported in our patent applications, the longer full length human Asp2 cDNA has 501 codons.[1] (Figure 3 of the patent applications.) As correctly reported in our 1999 patent applications, the transmembrane domain of this Asp2 clone spans approximately residues 455 to 477 of the full length Asp2 sequence.

7. Looking back, I believe that the inadvertent error in the 60/155,493 application occurred because our research team had performed some of the routine computer-aided analysis on a partial Asp2 sequence from the '438 clone, and reported the data from this analysis for the full length Asp2 clone in the patent application. The analysis of the partial sequence from the '438 clone indicated that the transmembrane domain corresponded approximately to residues 367-392 *of the partial sequence*. (See Exhibit A hereto, which is a computer-assisted analysis of Asp2 (clone '438) sequence for possible transmembrane domains, performed prior to September 24, 1998, which indicates a likely TM region at about 367-392 of the sequenced analyzed.) I believe that the numbers from this analysis *of the '438 partial sequence* were reported in the 60/155,493 application for the *full length* short form (Figure 2) of human Asp2.[2] Since the patent application reported the full length Asp2 sequences, the numbers that were generated using the '438 clone partial sequence should have been adjusted upward for the patent application, to account for the extra codons at the beginning of the full length clone that were missing from the '438 clone partial sequence

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1 An Asp2 splice variant described in our patent application has 476 codons by virtue of the internal deletion of 25 codons described above in paragraph 3. (Figure 2 of the patent applications.) As reported correctly our 1999 patent applications, the transmembrane domain of this sequence corresponds approximately to residues 430-452.

2 An upward adjustment of these numbers (by 25 codons) was used for the long form of Asp2 (Figure 3).

analyzed. But, through inadvertent error when preparing the patent application, this adjustment was not made.

8. In my opinion, this error would have been apparent to an average scientist in the field who evaluated the application, as would the proper correction of the error. In particular, it is commonly understood by molecular biologists that a transmembrane domain is characterized by a stretch of about 20-25 mostly hydrophobic amino acids. When a biologist read the application's teaching that Asp2 had a transmembrane domain near the carboxy-terminus and then examined the sequence to look for that transmembrane domain, it would have been readily apparent that the transmembrane domain was at about residues 455-477 (of Figure 3), and not residues 392-417.

III. Invention-related activity for Asp2 ΔTM polynucleotides and polypeptides.

9. The attorneys for Pharmacia & Upjohn have asked me to authenticate and discuss certain documents relating to our Asp2 invention.

10. Exhibit B hereto comprises excerpts from our U.S. Provisional Application No. 60/101,594. These excerpts establish that, on or before our filing date of September 24, 1998, we had possession of two human Asp2 cDNA and deduced Asp2 amino acid sequences (Figures 2 and 3) and determined various Asp2 structural features, including the presence of a transmembrane domain. It shows that we contemplated vectors and host cells for recombinant production of Asp2 polypeptides and enzymatically active polypeptide fragments (see, e.g., pp. 4, 5, and 9), and that we contemplated Asp2 antibodies (see, e.g., pp. 4, 12.) It shows that we contemplated expression of Asp2 in a variety of expression systems, including prokaryotes such as *E. coli* (pp. 9 and 10), yeasts such as *S. cerevisiae* (pp. 9, 11), and higher eukaryotes such as insect cell systems and mammalian systems, including COS cells, CHO cells, and human cells (see, e.g., pp. 9, 11-12).

11. Exhibit C hereto is a copy of a page from a Pharmacia & Upjohn interoffice memo from prior to our September 24, 1998, filing date, containing a report on the Human Asp2 project. Among other things, this except shows that, prior to September 24,

1998, we had engineered the Asp2 open reading frame (ORF) from the '438 and '269 clones to remove the transmembrane domains, and that we had inserted these ΔTM constructs into an *E. coli* expression vector pQE30.

12. Exhibit D hereto are copies of pages from a Pharmacia & Upjohn laboratory notebook. These pages establish that, prior to December 31, 1998, we had made a human Asp 2 ΔTM construct containing the DNA sequence coding for human Asp2 amino acids 1-454 (long form shown in Figure 3 of patent applications) in a baculovirus expression vector pVL 1393 (hu Asp 2 ΔTM pVL 1393) for expression in SF9 insect cells. This construct was sent for sequencing and the sequence was confirmed. Exhibit E hereto are copies of pages from a Pharmacia & Upjohn laboratory notebook. These pages establish that after December 31, 1998, we had made similar constructs with 6-histidine tags to facilitate protein purification.

13. Exhibit F hereto are copies of pages from Pharmacia & Upjohn laboratory notebooks which show that, prior to March 26, 1999, we had expressed human Asp2 ΔTM protein (without ß secretase enzyme activity) in *E. Coli* to make antibodies for use in testing of recombinant expression of human Asp2 ΔTM in other cell types.

14. Exhibit G hereto are copies of pages from a Pharmacia & Upjohn laboratory notebook which show that, prior to March 26, 1999, we had made, isolated, and scaled-up preparations of viral plaques for production of a human Asp2 ΔTM construct in SF9 insect cells.

15. Exhibit H hereto contains copies of pages from a Pharmacia & Upjohn laboratory notebook which show that, after December 31, 1998, and prior to June 15, 1999, and prior to September 23, 1999, the scale-up results from SF9 were analyzed. Exhibit I are copies of a Pharmacia and Upjohn laboratory notebook showing a gel depicting the results of such analysis. A clean band of human Asp2 ΔTM expressed protein was identified by Western blot as shown in the notebook. This band is believed to contain active human ΔTM Asp2 1-454 protein expressed in the SF9 system.

16. Exhibit J are copies of pages from a Pharmacia & Upjohn laboratory notebook showing that, after March 26, 1999, but prior to September 23, 1999, we excised the 1-454 Asp2 ΔTM coding segment from the pVL 1393 vector described above, inserted it into PIZ vector, and expressed this Asp2 ΔTM construct in High Five Cells. We tested this recombinant human Asp2 ΔTM protein and showed that it retained human Asp2 enzymatic activity. This work is also generally described in the patent applications that we filed on September 23, 1999, including PCT/US99/20881, U.S. Provisional Application No. 60/155,493, and U.S. Application Serial No. 09/404,133.

17. As shown in part by the representative documents referred to in the preceding paragraphs, during the period prior to September 24, 1998, until September 23, 1999, we were engaged in substantially continuous activity to make enzymatically active human Asp2 protein lacking a transmembrane domain, using materials and methods that we had contemplated in our September 24, 1998, patent application and/or had produced by that September 24, 1998 filing date.

IV. Certification

18. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: November 30, 2001

Michael J. Bienkowski

Michael Jerome Bienkowski, Ph.D.

Figure 3 Alignment of Prosite Aspartyl protease consensus sequence with active site motifs in Hu_Asp-2

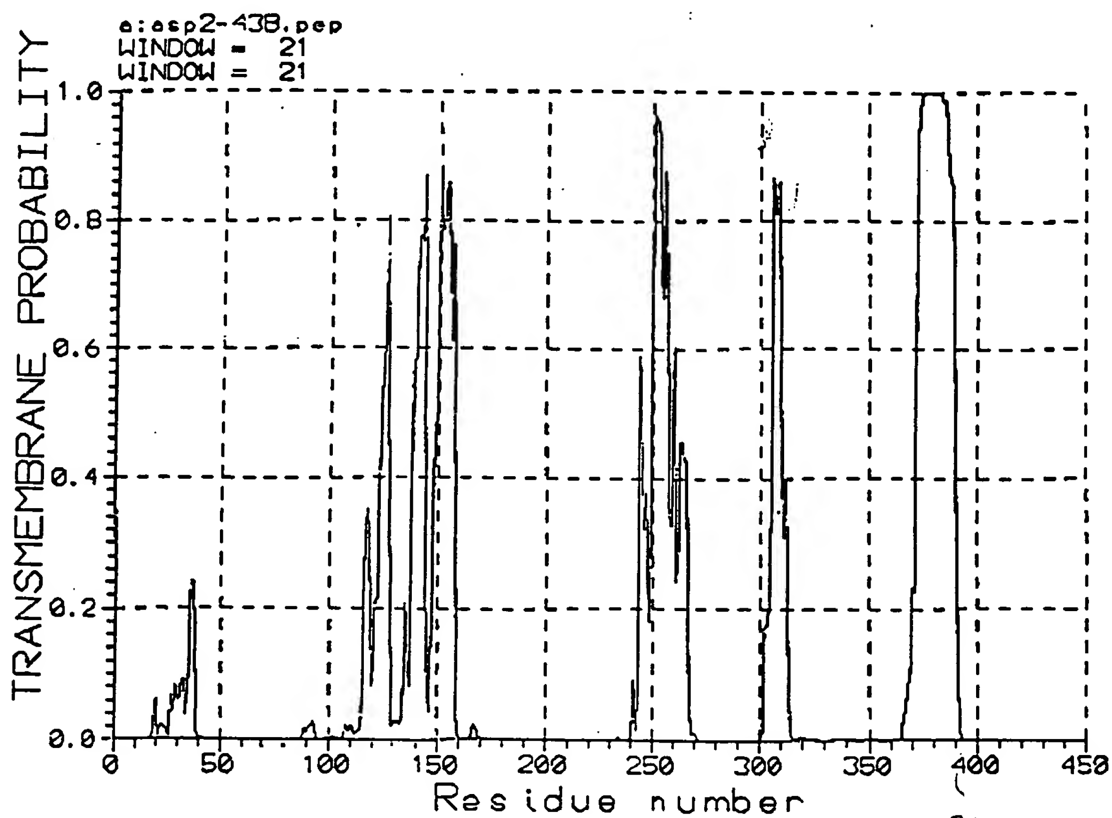
[LIVMFGAC]-[LIVMTADN]-[LIVFSA]-D-[ST]-G-[STAV]-[STAPDENQ]-X-[LIVMFSTNC]-X-[LIVMFGTA]

N-Terminal motif: ILVDTGSSNFAV

[LIVMFGAC]-[LIVMTADN]-[LIVFSA]-D-[ST]-G-[STAV]-[STAPDENQ]-X-[LIVMFSTNC]-X-[LIVMFGTA]

C-Terminal motif: SIVDSGTTNLRL

Figure 4



In a preferred embodiment, the nucleic acid molecules comprise a polynucleotide having a nucleotide sequence selected from the group consisting of residues 21-1290 of SEQ ID NO:1, encoding Hu-Asp1, residues 84-1325 of SEQ ID NO:3, encoding Hu-Asp2(a), and residues 84-1400 of SEQ ID NO:5, encoding Hu-Asp2(b). In another aspect, the invention provides an isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent conditions to a polynucleotide encoding Hu-Asp1, Hu-Asp2(a), Hu-Asp-2(b), or fragments thereof. European patent application EP 0 848 062 discloses a polypeptide referred to as "Asp 1," that bears substantial homology to Hu-Asp1, while international application WO 98/22597 discloses a polypeptide referred to as "Asp 2," that bears substantial homology to Hu-Asp2a.

The present invention also provides vectors comprising the isolated nucleic acid molecules of the invention, host cells into which such vectors have been introduced, and recombinant methods of obtaining a Hu-Asp1, Hu-Asp2(a), or Hu-Asp2(b) polypeptide comprising culturing the above-described host cell and isolating the relevant polypeptide.

In another aspect, the invention provides isolated Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b) polypeptides, as well as fragments thereof. In a preferred embodiment, the Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b) polypeptides have the amino acid sequence given in SEQ ID NO:2, SEQ ID NO:4, or SEQ ID NO:6, respectively. Isolated antibodies, both polyclonal and monoclonal, that bind specifically to any of the Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b) polypeptides of the invention are also provided.

The invention also provides a method for the identification of an agent that modulates the activity of any of Hu-Asp-1, Hu-Asp2(a), and Hu-Asp2(b).

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Figure 1 shows the nucleotide (SEQ ID NO:1) and predicted amino acid sequence (SEQ ID NO:2) of human Asp1.

Figure 2: Figure 2 shows the nucleotide (SEQ ID NO:3) and predicted amino acid sequence (SEQ ID NO:4) of human Asp2(a).

Figure 3: Figure 3 shows the nucleotide (SEQ ID NO:5) and predicted amino acid sequence (SEQ ID NO:6) of human Asp2(b). The predicted transmembrane domain of Hu-Asp2(b) is enclosed in brackets.

Figure 4: Figure 4 shows the sequence (SEQ ID NO:) of APP695 C-terminus after addition of the di-Lys motif using "patch" PCR.

DETAILED DESCRIPTION OF THE INVENTION

The present invention describes a method to scan gene data bases for the simple active site motif characteristic of aspartyl proteases. Eukaryotic aspartyl proteases such as pepsin and renin possess a two domain structure which folds to bring two aspartyl residues into proximity within the active site. These are embedded in the short tripeptide motif DTG, or more rarely, DSG. Most aspartyl proteases occur as proenzyme whose N-terminus must be cleaved for activation. The DTG or DSG active site motif appears at about residue 65-70 in the proenzyme (prorenin, pepsinogen), but at about residue 25-30 in the active enzyme after cleavage of the N-terminal prodomain. The limited length of the active site motif makes it difficult to search collections of short, expressed sequence tags (EST) for novel aspartyl proteases. EST sequences typically average 250 nucleotides or less, and so would encode 80-90 amino acid residues or less. That would be too short a sequence to span the two active site motifs. The preferred method is to scan data bases of hypothetical or assembled protein coding sequences. The present invention describes a computer method to identify candidate aspartyl proteases in protein sequence data bases. The method was used to identify seven candidate aspartyl protease sequences in the Caenorhabditis elegans genome. These sequences were then used to identify by homology search Hu-Asp1 and two alternative splice variants of Hu-Asp2, designated herein as Hu-Asp2(a) and Hu-Asp2(b).

In another embodiment, the invention provides isolated nucleic acid molecules comprising a polynucleotide encoding a polypeptide selected from the group consisting of human aspartyl protease 1 (Hu-Asp1) and two alternative splice variants of human aspartyl protease 2 (Hu-Asp2), designated herein as Hu-Asp2(a) and Hu-Asp2(b). As used herein, all references to "Hu-Asp2" should be understood to refer to both Hu-Asp2(a) and Hu-Asp2(b). Hu-Asp1 is expressed most abundantly inpancreas and prostate tissues, while Hu-Asp2(a) and Hu-Asp2(b) are expressed most abundantly in pancreas and brain tissues, with low levels of expression observed in all other tissues examined except thymus and PBLs. The invention also provides isolated Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b) polypeptides, as well as fragments thereof which exhibit aspartyl protease activity.

The predicted amino acid sequences of Hu-Asp1, Hu-Asp2(a) and Hu-Asp2(b) share significant homology with previously identified mammalian aspartyl proteases such as pepsinogen A, pepsinogen B, cathepsin D, cathepsin E, and renin. P.B.Szecs, *Scand. J. Clin. Lab. Invest.* 52:(Suppl. 210 5-22 (1992)). These enzymes are characterized by the presence of a duplicated DTG/DSG sequence motif. The Hu-Asp1 and HuAsp2

may be similar to or significantly different from a native Hu-Asp polypeptide in molecular weight and glycosylation pattern. Expression of Hu-Asp in bacterial expression systems will provide non-glycosylated Hu-Asp.

The polypeptides of the present invention are preferably provided in an isolated form, and preferably are substantially purified. Hu-Asp polypeptides may be recovered and purified from recombinant cell cultures by well-known methods, including ammonium sulfate or ethanol precipitation, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. In a preferred embodiment, high performance liquid chromatography (HPLC) is employed for purification.

The present invention also relates to vectors comprising the polynucleotide molecules of the invention, as well as host cell transformed with such vectors. Any of the polynucleotide molecules of the invention may be joined to a vector, which generally includes a selectable marker and an origin of replication, for propagation in a host. Because the invention also provides Hu-Asp polypeptides expressed from the polynucleotide molecules described above, vectors for the expression of Hu-Asp are preferred. The vectors include DNA encoding any of the Hu-Asp polypeptides described above or below, operably linked to suitable transcriptional or translational regulatory sequences, such as those derived from a mammalian, microbial, viral, or insect gene. Examples of regulatory sequences include transcriptional promoters, operators, or enhancers, mRNA ribosomal binding sites, and appropriate sequences which control transcription and translation. Nucleotide sequences are operably linked when the regulatory sequence functionally relates to the DNA encoding Hu-Asp. Thus, a promoter nucleotide sequence is operably linked to a Hu-Asp DNA sequence if the promoter nucleotide sequence directs the transcription of the Hu-Asp sequence.

Selection of suitable vectors to be used for the cloning of polynucleotide molecules encoding Hu-Asp, or for the expression of Hu-Asp polypeptides, will of course depend upon the host cell in which the vector will be transformed, and, where applicable, the host cell from which the Hu-Asp polypeptide is to be expressed. Suitable host cells for expression of Hu-Asp polypeptides include prokaryotes, yeast, and higher eukaryotic cells, each of which is discussed below.

The Hu-Asp polypeptides to be expressed in such host cells may also be fusion proteins which include regions from heterologous proteins. Such regions may be included to

allow, *e.g.*, secretion, improved stability, or facilitated purification of the polypeptide. For example, a sequence encoding an appropriate signal peptide can be incorporated into expression vectors. A DNA sequence for a signal peptide (secretory leader) may be fused in-frame to the Hu-Asp sequence so that Hu-Asp is translated as a fusion protein comprising the signal peptide. A signal peptide that is functional in the intended host cell promotes extracellular secretion of the Hu-Asp polypeptide. Preferably, the signal sequence will be cleaved from the Hu-Asp polypeptide upon secretion of Hu-Asp from the cell. Non-limiting examples of signal sequences that can be used in practicing the invention include the yeast I-factor and the honeybee melatin leader in sf9 insect cells.

In a preferred embodiment, the Hu-Asp polypeptide will be a fusion protein which includes a heterologous region used to facilitate purification of the polypeptide. Many of the available peptides used for such a function allow selective binding of the fusion protein to a binding partner. For example, the Hu-Asp polypeptide may be modified to comprise a peptide to form a fusion protein which specifically binds to a binding partner, or peptide tag. Non-limiting examples of such peptide tags include the 6-His tag, thioredoxin tag, hemaglutinin tag, GST tag, and OmpA signal sequence tag. As will be understood by one of skill in the art, the binding partner which recognizes and binds to the peptide may be any molecule or compound including metal ions (*e.g.*, metal affinity columns), antibodies, or fragments thereof, and any protein or peptide which binds the peptide, such as the FLAG tag.

Suitable host cells for expression of Hu-Asp polypeptides include prokaryotes, yeast, and higher eukaryotic cells. Suitable prokaryotic hosts to be used for the expression of Hu-Asp include bacteria of the genera *Escherichia, Bacillus, and Salmonella,* as well as members of the genera *Pseudomonas, Streptomyces,* and *Staphylococcus.* For expression in, *e.g.*, *E. coli,* a Hu-Asp polypeptide may include an N-terminal methionine residue to facilitate expression of the recombinant polypeptide in a prokaryotic host. The N-terminal Met may optionally then be cleaved from the expressed Hu-Asp polypeptide.

Expression vectors for use in prokaryotic hosts generally comprise one or more phenotypic selectable marker genes. Such genes generally encode, *e.g.*, a protein that confers antibiotic resistance or that supplies an auxotrophic requirement. A wide variety of such vectors are readily available from commercial sources. Examples include pSPORT vectors, pGEM vectors (Promega), pPROEX vectors (LTI, Bethesda, MD), Bluescript vectors (Stratagene), and pQE vectors (Qiagen).

Hu-Asp may also be expressed in yeast host cells from genera including *Saccharomyces, Pichia,* and *Kluveromyces.* Preferred yeast hosts are *S. cerevisiae* and *P. pastoris.* Yeast vectors will often contain an origin of replication sequence from a 2T yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination, and a selectable marker gene. Vectors replicable in both yeast and *E. coli* (termed shuttle vectors) may also be used. In addition to the above-mentioned features of yeast vectors, a shuttle vector will also include sequences for replication and selection in *E. coli*. Direct secretion of Hu-Asp polypeptides expressed in yeast hosts may be accomplished by the inclusion of nucleotide sequence encoding the yeast I-factor leader sequence at the 5' end of the Hu-Asp-encoding nucleotide sequence.

Insect host cell culture systems may also be used for the expression of Hu-Asp polypeptides. In a preferred embodiment, the Hu-Asp polypeptides of the invention are expressed using a baculovirus expression system (*see* Example 3). Further information regarding the use of baculovirus systems for the expression of heterologous proteins in insect cells are reviewed by Luckow and Summers, *Bio/Technology* 6:47 (1988).

In another preferred embodiment, the Hu-Asp polypeptide is expressed in mammalian host cells. Non-limiting examples of suitable mammalian cell lines include the COS-7 line of monkey kidney cells (Gluzman *et al., Cell* 23:175 (1981)) and Chinese hamster ovary (CHO) cells. Preferably, human embryonic kidney cell line 293 is used for expression of Hu-Asp proteins.

The choice of a suitable expression vector for expression of the Hu-Asp polypeptides of the invention will of course depend upon the specific mammalian host cell to be used, and is within the skill of the ordinary artisan. Examples of suitable expression vectors include pcDNA3 (Invitrogen) and pSVL (Pharmacia Biotech). A preferred vector for expression of Hu-Asp polypeptides is pBK-CMV (Stratagene). Expression vectors for use in mammalian host cells may include transcriptional and translational control sequences derived from viral genomes. Commonly used promoter sequences and enhancer sequences which may be used in the present invention include, but are not limited to, those derived from human cytomegalovirus (CMV), Adenovirus 2, Polyoma virus, and Simian virus 40 (SV40). Methods for the construction of mammalian expression vectors are disclosed, for example, in Okayama and Berg (*Mol. Cell. Biol.* 3:280 (1983)); Cosman *et al.* (*Mol. Immunol.*

23:935 (1986)); Cosman *et al.* (*Nature 312*:768 (1984)); EP-A-0367566; and WO 91/18982.

The polypeptides of the present invention may also be used to raise polyclonal and monoclonal antibodies, which are useful in diagnostic assays for detecting Hu-Asp polypeptide expression. Such antibodies may be prepared by conventional techniques. See, for example, *Antibodies: A Laboratory Manual*, Harlow and Land (eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1988); *Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses*, Kennet *et al.* (eds.), Plenum Press, New York (1980).

The Hu-Asp nucleic acid molecules of the present invention are also valuable for chromosome identification, as they can hybridize with a specific location on a human chromosome. Hu-Asp1 has been localized to chromosome 21, while Hu-Asp2 has been localized to chromosome 11. There is a current need for identifying particular sites on the chromosome, as few chromosome marking reagents based on actual sequence data (repeat polymorphisms) are presently available for marking chromosomal location. Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. The relationship between genes and diseases that have been mapped to the same chromosomal region can then be identified through linkage analysis, wherein the coinheritance of physically adjacent genes is determined. Whether a gene appearing to be related to a particular disease is in fact the cause of the disease can then be determined by comparing the nucleic acid sequence between affected and unaffected individuals.

In another embodiment, the invention relates to a method for the identification of an agent that increases the activity of a Hu-Asp polypeptide selected from the group consisting of Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b), the method comprising

(a) determining the activity of said Hu-Asp polypeptide in the presence of a test agent and in the absence of a test agent; and

(b) comparing the activity of said Hu-Asp polypeptide determined in the presence of said test agent to the activity of said Hu-Asp polypeptide determined in the absence of said test agent;

whereby a higher level of activity in the presence of said test agent than in the absence of said test agent indicates that said test agent has increased the activity of said Hu-Asp polypeptide.

FIGURE 2

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         10                            30                            50
ATGGCCCAAGCCCTGCCCTGGCTCCTGCTGTGGATGGGCGCGGGAGTGCTGCCTGCCCAC
M  A  Q  A  L  P  W  L  L  L  W  M  G  A  G  V  L  P  A  H
         70                            90                           110
GGCACCCAGCACGGCATCCGGCTGCCCCTGCGCAGCGGCCTGGGGGGCGCCCCCCTGGGG
G  T  Q  H  G  I  R  L  P  L  R  S  G  L  G  G  A  P  L  G
        130                           150                           170
CTGCGGCTGCCCCGGGAGACCGACGAAGAGCCCGAGGAGCCCGGCCGGAGGGGCAGCTTT
L  R  L  P  R  E  T  D  E  E  P  E  E  P  G  R  R  G  S  F
        190                           210                           230
GTGGAGATGGTGGACAACCTGAGGGGCAAGTCGGGGCAGGGCTACTACGTGGAGATGACC
V  E  M  V  D  N  L  R  G  K  S  G  Q  G  Y  Y  V  E  M  T
        250                           270                           290
GTGGGCAGCCCCCCGCAGACGCTCAACATCCTGGTGGATACAGGCAGCAGTAACTTTGCA
V  G  S  P  P  Q  T  L  N  I  L  V  D  T  G  S  S  N  F  A
        310                           330                           350
GTGGGTGCTGCCCCCCACCCCTTCCTGCATCGCTACTACCAGAGGCAGCTGTCCAGCACA
V  G  A  A  P  H  P  F  L  H  R  Y  Y  Q  R  Q  L  S  S  T
        370                           390                           410
TACCGGGACCTCCGGAAGGGTGTGTATGTGCCCTACACCCAGGGCAAGTGGGAAGGGGAG
Y  R  D  L  R  K  G  V  Y  V  P  Y  T  Q  G  K  W  E  G  E
        430                           450                           470
CTGGGCACCGACCTGGTAAGCATCCCCCATGGCCCCAACGTCACTGTGCGTGCCAACATT
L  G  T  D  L  V  S  I  P  H  G  P  N  V  T  V  R  A  N  I
        490                           510                           530
GCTGCCATCACTGAATCAGACAAGTTCTTCATCAACGGCTCCAACTGGGAAGGCATCCTG
A  A  I  T  E  S  D  K  F  F  I  N  G  S  N  W  E  G  I  L
        550                           570                           590
GGGCTGGCCTATGCTGAGATTGCCAGGCTTTGTGGTGCTGGCTTCCCCCTCAACCAGTCT
G  L  A  Y  A  E  I  A  R  L  C  G  A  G  F  P  L  N  Q  S
        610                           630                           650
GAAGTGCTGGCCTCTGTCGGAGGGAGCATGATCATTGGAGGTATCGACCACTCGCTGTAC
E  V  L  A  S  V  G  G  S  M  I  I  G  G  I  D  H  S  L  Y
        670                           690                           710
ACAGGCAGTCTCTGGTATACACCCATCCGGCGGGAGTGGTATTATGAGGTGATCATTGTG
T  G  S  L  W  Y  T  P  I  R  R  E  W  Y  Y  E  V  I  I  V
        730                           750                           770
CGGGTGGAGATCAATGGACAGGATCTGAAAATGGACTGCAAGGAGTACAACTATGACAAG
R  V  E  I  N  G  Q  D  L  K  M  D  C  K  E  Y  N  Y  D  K
        790                           810                           830
AGCATTGTGGACAGTGGCACCACCAACCTTCGTTTGCCCAAGAAAGTGTTTGAAGCTGCA
S  I  V  D  S  G  T  T  N  L  R  L  P  K  K  V  F  E  A  A
        850                           870                           890
GTCAAATCCATCAAGGCAGCCTCCTCCACGGAGAAGTTCCCTGATGGTTTCTGGCTAGGA
V  K  S  I  K  A  A  S  S  T  E  K  F  P  D  G  F  W  L  G
        910                           930                           950
GAGCAGCTGGTGTGCTGGCAAGCAGGCACCACCCCTTGGAACATTTTCCCAGTCATCTCA
E  Q  L  V  C  W  Q  A  G  T  T  P  W  N  I  F  P  V  I  S
        970                           990                          1010
CTCTACCTAATGGGTGAGGTTACCAACCAGTCCTTCCGCATCACCATCCTTCCGCAGCAA
L  Y  L  M  G  E  V  T  N  Q  S  F  R  I  T  I  L  P  Q  Q
       1030                          1050                          1070
TACCTGCGGCCAGTGGAAGATGTGGCCACGTCCCAAGACGACTGTTACAAGTTTGCCATC
Y  L  R  P  V  E  D  V  A  T  S  Q  D  D  C  Y  K  F  A  I
       1090                          1110                          1130
TCACAGTCATCCACGGGCACTGTTATGGGAGCTGTTATCATGGAGGGCTTCTACGTTGTC
S  Q  S  S  T  G  T  V  M  G  A  V  I  M  E  G  F  Y  V  V
       1150                          1170                          1190
TTTGATCGGGCCCGAAAACGAATTGGCTTTGCTGTCAGCGCTTGCCATGTGCACGATGAG
F  D  R  A  R  K  R  I  G  F  A  V  S  A  C  H  V  H  D  E
       1210                          1230                          1250
TTCAGGACGGCAGCGGTGGAAGGCCCTTTTGTCACCTTGGACATGGAAGACTGTGGCTAC
F  R  T  A  A  V  E  G  P  F  V  T  L  D  M  E  D  C  G  Y
       1270                          1290                          1310
AACATTCCACAGACAGATGAGTCAACCCTCATGACCATAGCCTATGTCATGGCTGCCATC
N  I  P  Q  T  D  E  S  T  L  M  T  I  A  Y  V  M  A  A  I
       1330                          1350                          1370
TGCGCCCTCTTCATGCTGCCACTCTGCCTCATGGTGTGTCAGTGGCGCTGCCTCCGCTGC
```

```
C  A  L  F  M  L  P  L  C  L  M  V  C  Q  W  R  C  L  R  C
         1390                          1410                          1430
CTGCGCCAGCAGCATGATGACTTTGCTGATGACATCTCCCTGCTGAAGTGAGGAGGCCCA
L  R  Q  Q  H  D  D  F  A  D  D  I  S  L  L  K
         1450                          1470                          1490
TGGGCAGAAGATAGAGATTCCCCTGGACCACACCTCCGTGGTTCACTTTGGTCACAAGTA
         1510                          1530                          1550
GGAGACACAGATGGCACCTGTGGCCAGAGCACCTCAGGACCCTCCCCACCCACCAAATGC
         1570                          1590                          1610
CTCTGCCTTGATGGAGAAGGAAAAGGCTGGCAAGGTGGGTTCCAGGGACTGTACCTGTAG
         1630                          1650                          1670
GAAACAGAAAAGAGAAGAAAGAAGCACTCTGCTGGCGGGAATACTCTTGGTCACCTCAAA
         1690                          1710                          1730
TTTAAGTCGGGAAATTCTGCTGCTTGAAACTTCAGCCCTGAACCTTTGTCCACCATTCCT
         1750                          1770                          1790
TTAAATTCTCCAACCCAAAGTATTCTTCTTTTCTTAGTTTCAGAAGTACTGGCATCACAC
         1810                          1830                          1850
GCAGGTTACCTTGGCGTGTGTCCCTGTGGTACCCTGGCAGAGAAGAGACCAAGCTTGTTT
         1870                          1890                          1910
CCCTGCTGGCCAAAGTCAGTAGGAGAGGATGCACAGTTTGCTATTTGCTTTAGAGACAGG
         1930                          1950                          1970
GACTGTATAAACAAGCCTAACATTGGTGCAAAGATTGCCTCTTGAAAAAAAAAAAAA
```

FIGURE 3

```
10                  30                  50
ATGGCCCAAGCCCTGCCCTGGCTCCTGCTGTGGATGGGCGCGGGAGTGCTGCCTGCCCAC
M  A  Q  A  L  P  W  L  L  L  W  M  G  A  G  V  L  P  A  H
70                  90                  110
GGCACCCAGCACGGCATCCGGCTGCCCCTGCGCAGCGGCCTGGGGGGCGCCCCCCTGGGG
G  T  Q  H  G  I  R  L  P  L  R  S  G  L  G  G  A  P  L  G
130                 150                 170
CTGCGGCTGCCCCGGGAGACCGACGAAGAGCCCGAGGAGCCCGGCCGGAGGGGCAGCTTT
L  R  L  P  R  E  T  D  E  E  P  E  E  P  G  R  R  G  S  F
190                 210                 230
GTGGAGATGGTGGACAACCTGAGGGGCAAGTCGGGGCAGGGCTACTACGTGGAGATGACC
V  E  M  V  D  N  L  R  G  K  S  G  Q  G  Y  Y  V  E  M  T
250                 270                 290
GTGGGCAGCCCCCCGCAGACGCTCAACATCCTGGTGGATACAGGCAGCAGTAACTTTGCA
V  G  S  P  P  Q  T  L  N  I  L  V  D  T  G  S  S  N  F  A
310                 330                 350
GTGGGTGCTGCCCCCCACCCCTTCCTGCATCGCTACTACCAGAGGCAGCTGTCCAGCACA
V  G  A  A  P  H  P  F  L  H  R  Y  Y  Q  R  Q  L  S  S  T
370                 390                 410
TACCGGGACCTCCGGAAGGGTGTGTATGTGCCCTACACCCAGGGCAAGTGGGAAGGGGAG
Y  R  D  L  R  K  G  V  Y  V  P  Y  T  Q  G  K  W  E  G  E
430                 450                 470
CTGGGCACCGACCTGGTAAGCATCCCCCATGGCCCCAACGTCACTGTGCGTGCCAACATT
L  G  T  D  L  V  S  I  P  H  G  P  N  V  T  V  R  A  N  I
490                 510                 530
GCTGCCATCACTGAATCAGACAAGTTCTTCATCAACGGCTCCAACTGGGAAGGCATCCTG
A  A  I  T  E  S  D  K  F  F  I  N  G  S  N  W  E  G  I  L
550                 570                 590
GGGCTGGCCTATGCTGAGATTGCCAGGCCTGACGACTCCCTGGAGCCTTTCTTTGACTCT
G  L  A  Y  A  E  I  A  R  P  D  D  S  L  E  P  F  F  D  S
610                 630                 650
CTGGTAAAGCAGACCCACGTTCCCAACCTCTTCTCCCTGCAGCTTTGTGGTGCTGGCTTC
L  V  K  Q  T  H  V  P  N  L  F  S  L  Q  L  C  G  A  G  F
670                 690                 710
CCCCTCAACCAGTCTGAAGTGCTGGCCTCTGTCGGAGGGAGCATGATCATTGGAGGTATC
P  L  N  Q  S  E  V  L  A  S  V  G  G  S  M  I  I  G  G  I
730                 750                 770
GACCACTCGCTGTACACAGGCAGTCTCTGGTATACACCCATCCGGCGGGAGTGGTATTAT
D  H  S  L  Y  T  G  S  L  W  Y  T  P  I  R  R  E  W  Y  Y
790                 810                 830
GAGGTCATCATTGTGCGGGTGGAGATCAATGGACAGGATCTGAAAATGGACTGCAAGGAG
E  V  I  I  V  R  V  E  I  N  G  Q  D  L  K  M  D  C  K  E
850                 870                 890
TACAACTATGACAAGAGCATTGTGGACAGTGGCACCACCAACCTTCGTTTGCCCAAGAAA
Y  N  Y  D  K  S  I  V  D  S  G  T  T  N  L  R  L  P  K  K
910                 930                 950
GTGTTTGAAGCTGCAGTCAAATCCATCAAGGCAGCCTCCTCCACGGAGAAGTTCCCTGAT
V  F  E  A  A  V  K  S  I  K  A  A  S  S  T  E  K  F  P  D
970                 990                 1010
GGTTTCTGGCTAGGAGAGCAGCTGGTGTGCTGGCAAGCAGGCACCACCCCTTGGAACATT
G  F  W  L  G  E  Q  L  V  C  W  Q  A  G  T  T  P  W  N  I
1030                1050                1070
TTCCCAGTCATCTCACTCTACCTAATGGGTGAGGTTACCAACCAGTCCTTCCGCATCACC
F  P  V  I  S  L  Y  L  M  G  E  V  T  N  Q  S  F  R  I  T
1090                1110                1130
ATCCTTCCGCAGCAATACCTGCGGCCAGTGGAAGATGTGGCCACGTCCCAAGACGACTGT
I  L  P  Q  Q  Y  L  R  P  V  E  D  V  A  T  S  Q  D  D  C
1150                1170                1190
TACAAGTTTGCCATCTCACAGTCATCCACGGGCACTGTTATGGGAGCTGTTATCATGGAG
Y  K  F  A  I  S  Q  S  S  T  G  T  V  M  G  A  V  I  M  E
1210                1230                1250
GGCTTCTACGTTGTCTTTGATCGGGCCCGAAAACGAATTGGCTTTGCTGTCAGCGCTTGC
G  F  Y  V  V  F  D  R  A  R  K  R  I  G  F  A  V  S  A  C
1270                1290                1310
CATGTGCACGATGAGTTCAGGACGGCAGCGGTGGAAGGCCCTTTTGTCACCTTGGACATG
H  V  H  D  E  F  R  T  A  A  V  E  G  P  F  V  T  L  D  M
1330                1350                1370
GAAGACTGTGGCTACAACATTCCACAGACAGATGAGTCAACCCTCATGACCATAGCCTAT
```

```
                                          T   D   E   S   T   L   M   T   I   A   Y
E   D   C   G   Y   N   I   P   Q                                  1430
       1390                          1410
GTCATGGCTGCCATCTGCGCCCTCTTCATGCTGCCACTCTGCCTCATGGTGTGTCAGTGG
V   M   A   A   I   C   A   L   F   M   L   P   L   C   L   M   V   C   Q   W
       1450                          1470                          1490
CGCTGCCTCCGCTGCCTGCGCCAGCAGCATGATGACTTTGCTGATGACATCTCCCTGCTG
R   C   L   R   C   L   R   Q   Q   H   D   D   F   A   D   D   I   S   L   L
       1510                          1530                          1550
AAGTGAGGAGGCCCATGGGCAGAAGATAGAGATTCCCCTGGACCACACCTCCGTGGTTCA
K
       1570                          1590                          1610
CTTTGGTCACAAGTAGGAGACACAGATGGCACCTGTGGCCAGAGCACCTCAGGACCCTCC
       1630                          1650                          1670
CCACCCACCAAATGCCTCTGCCTTGATGGAGAAGGAAAAGGCTGGCAAGGTGGGTTCCAG
       1690                          1710                          1730
GGACTGTACCTGTAGGAAACAGAAAAGAGAAGAAAGAAGCACTCTGCTGGCGGGAATACT
       1750                          1770                          1790
CTTGGTCACCTCAAATTTAAGTCGGGAAATTCTGCTGCTTGAAACTTCAGCCCTGAACCT
       1810                          1830                          1850
TTGTCCACCATTCCTTTAAATTCTCCAACCCAAAGTATTCTTCTTTTCTTAGTTTCAGAA
       1870                          1890                          1910
GTACTGGCATCACACGCAGGTTACCTTGGCGTGTGTCCCTGTGGTACCCTGGCAGAGAAG
       1930                          1950                          1970
AGACCAAGCTTGTTTCCCTGCTGGCCAAAGTCAGTAGGAGAGGATGCACAGTTTGCTATT
       1990                          2010                          2030
TGCTTTAGAGACAGGGACTGTATAAACAAGCCTAACATTGGTGCAAAGATTGCCTCTTGA
       2050                          2070
ATTAAAAAAAAAAAAAAAAAAAAAAAAAA
```

coding sequence of Hu_Asp-1 has been prepared and the predicted amino acid sequence, aligned with both the short and long forms of Hu_Asp-2, is attached. This splice variant of Hu_Asp-1 encodes a 521 amino acid polypeptide including a 27 residue signal peptide so the pro-form of the enzyme contains 76 amino acid residues upstream of the first active site motif. This upstream sequence also contains a third DSG motif. Alignment of the sequence surrounding this upstream DSG with the ProSite motif for aspartyl proteases revealed a poor match while the other two DTG/DSG motifs showed a good match. Alignment, with Hu_Asp-2 sequences using the Clustal W algorithm highlights two major differences between Hu_Asp-1 and Hu_Asp-2; the NH_2 terminal extension in Hu_Asp-1 is much longer and that Hu_Asp-1 appears to be more like the long form of Hu_Asp-2. The longest stretches of amino acid identity align with the two aspartyl protease active site motifs although other areas of conservation are also scored.

Finally, the Hu_Asp-1 gene was localized to human Chromosome 21 by hybridization to a Southern blot containing a series of mouse/human or hamster/human somatic cell hybrids (attached).

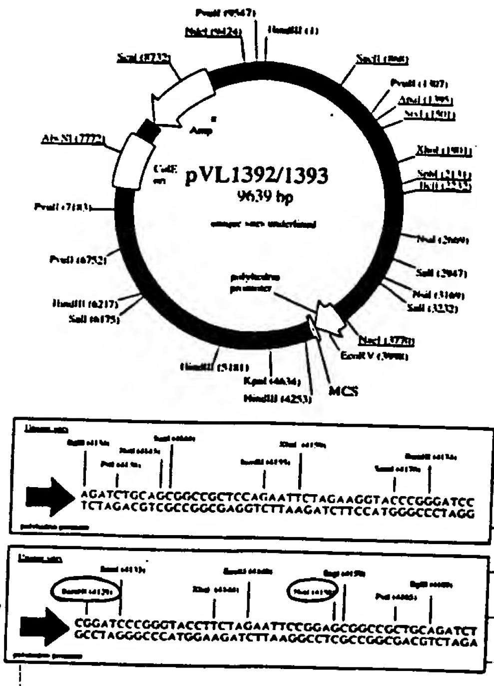
Hu_Asp-2, Mary provided an inventory of the expression constructs for Hu_Asp-2 (attached). The entire ORF of both the short (438) and long forms (269) of Hu_Asp-2 have be engineered into the mammalian cell expression vector pBK-CMV. Also, both the short

and long forms, with the COOH-terminal transmembrane domain deleted, have been prepared as NH_2 terminal 6His-fusions in the *E. coli* expression vector pQE30. Finally, the entire ORF from the short form of Hu_Asp-2 has been cloned downstream of the ecdysone-inducible promoter in the vector pIND and in a polycistronic fusion with GFP (pIRESGFP) for mammalian cell expression studies.

Hu_Asp-3 and Hu_Asp-4— Queries of the LifeSeq Assembled database with the sequences of either Hu_Asp-1 or Hu_Asp-2 identified (1) gene bins with exact matches to the query sequences, (2) gene bins matching the 5 known human aspartyl proteases [pepsinogen A, pepsinogen C, cathepsin D, cathepsin E and renin], and (3) three gene bins with significant homology [242842, 242824, 39511], in descending order of significance. Translation of the longest assembled templates contained within these gene bins revealed that they each encoded polypeptides containing the duplicated active site motif that is the hallmark of mammalian aspartyl proteases. Alignment of the predicted amino acid sequences for templates 451054.3 and 451034.4 showed that they were very similar with approximately 90% sequence identity at the amino acid level (attached). Template 126360 was most related to 451054.3 and 451034.4, with approximately 70% shared identity. Consistent with the nomenclature initiated previously, the genes represented by Incyte templates 451054.3, 451034.4, and 126360 are referred to as Hu_Asp-3, Hu_Asp-4a and Hu_Asp-5, respectively. Template 451034.2 appeared to be a splice variant of 450134.4 with a 25 amino acid (75 bp) insertion near the CO_2H-terminus (data not shown). The cDNAs that defined the 5'-most sequence of each of these templates were identified, obtained for sequence analysis and determination of the tissue distribution of expression of transcripts derived from these genes. The Hu_Asp-3 probe visualized a single 1.6 kb transcript that showed a limited expression pattern that was expressed at the highest levels in lung, immunological tissues (spleen, thymus and PBLs), and kidney (attached). No expression of Hu_Asp-3 transcripts was detected in whole brain while a weak signal was observed in several brain regions including the medulla, spinal cord and putamen (attached). These results were consistent with the expression pattern determined by EST sequencing in LifeSeq Assembled (39 ESTs) which indicated highest expression in the hematopoietic/imm category (41%) and the nervous category being the second highest (16%). The Hu_Asp-4 p visualized a similar pattern of transcript size and abundance except that the signal was mos in lung tissue. No transcripts were detected in either whole brain or selected brain regions conditions used in these experiments. A survey of expression using LifeSeq Assembled (1 indicated that 93% of the ESTs that comprise the Hu_Asp-4 template were derived from r

Asp2 → Baculovirus Expression

Engineer the pre-pro form of Asp2 ± TM for expression in Baculovirus using the Vector pVL1393



MCS pVL1392

MCS pVL1393

BAM | Kozak

GGATCC | GCC ACC ATG GCC ...

G S | S T M A Q A L ...

PCR: 100ng pcDNA3.1hygro Asp2 ® 1 µl
8 µl dNTPs
5 Pwo buf
1.5 Asp2BAM
(x2) or Asp2not-tm — 1.5 Asp2-not
1 Pwo I
32 H_2O

15 cycles

Extract, ppt. Digest w/ 83 µl H_2O
10 µl 10X #3
4 µl Bam
3 µl Not
37°C o/N

asp2Bam	CGC TTT GGA TCC GCC ACC ATG GCC CAA GCC CTG CCC TGG	BAM (Kozak) S T M A Q A L P W
asp2not-tm	CGC TTT GCGGCCGC CTA TGA CTC ATC TGT CTG TGG AAT GTT G	NOT * S E D T Q = reverse complement
asp2not	CGC TTT GCGGCCGC TCA CTT CAG CAG GGA GAT GTC ATC	NOT * K L L S I = reverse complement

Run 1% prep gel.

Geneclean frags.
Set up ligation O/N

(1)
Asp2/BAM-Not (100ng) = 1 µl
pVL1393 (160ng) 4 µl
8.5 µl + 1 µl

(2)
Asp2-tm/BAM Not 130ng = 2 µl
pVL1393 160ng = 4 µl

14°C o/N

Read and understood by me SW

Date

Tf DH5α w/ 2μl Plate on LB+Amp

Pick CFUs # ~~[illegible]~~ for long form — tm had no CFUs (#1-8)
PCR w/ Asp2-1 → Asp2-3
See p. 114 — looks good! #7
Plate total — tm tf

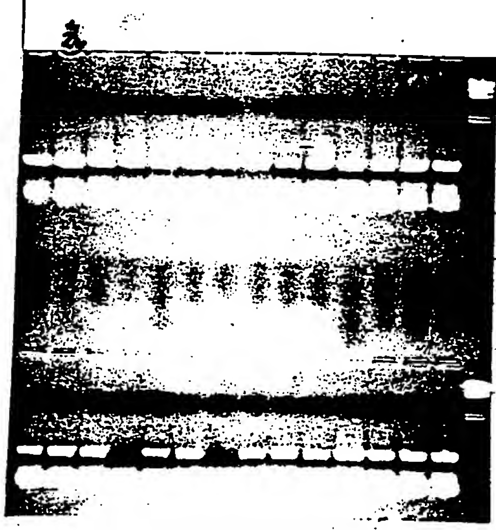
No — tm tf's ∴ ck frag o/N @ gel — lighter than expect — Set up new ligation.

— tm ligation: 2μl pVL1393 (80ng)
6μl Asp2 — tm (~55ng) } 14°C o/N
1μl 10X
1μl ligase

Tf DH5α w/ 2μl plate on LB-Amp
Pick 28 CFUs & PCR w/ Asp2-1 & Asp2-4 (427bp)

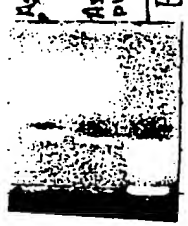
Asp2-tm pVL1393 Bug PCR
Asp2-1 → 2-4

Pick #2 for Cs prep - MJB



Harvested Cs preps - lots of debris in tubes
Extract, dialyze etc
Conc by OD: Asp2 pVL1393 = 1.37μg/μl
Asp2 ΔTM pVL1393 = 0.93μg/μl

Test digest w/ BAM + Not @ 37°C o/N
Given to D. Fisher for Baculo expression & Roger for Seq. confirmation



Seq of ± TM constructs is correct (1 nt change that is silent)

Read and understood by me

Date

SW

Generation of 6-His tagged Asp2 Baculo Constructs

- We have previously prepared Asp2 ± TM (full length - signal, pro ct) in the baculo expression vector pVL1393.

- We have recieved the first clones for Asp1+TM & Asp2+TM. Asp1 has been analyzed by PAGE & Western. There is a ton of protein being produced & is associated with the Sf9 cells. At this stage we can't say if the signal sequence is being clipped, if it's membrane associated or free in the cytosol.

- Anticipating difficulity in the purification of a non-secreted, non-tagged protein. I will design primers that incorporate a C-term 6 His. The primers will be as pag #105 notebook 31942 w/ 6 His added.

Asp2not-tmHis : CgcTTT gcggccgc (NOT I) CTA (*) -(ATg)x6 (6His) -TgA (S) -CTC (E)
ATC-TgT-CTg-Tgg-AAT-gTT-g

Asp2not His : CgcTTT gcggccgC (NOT) -TCA (*) -(ATg)x6-CTT (K) -CAg (L) -CAg (L) -ggA (S) -gAT (I) -
gTC-ATC

ASP2NOT-TMHIS	27629-001	60	DST	0.20	18515.0	91.5	32.8	24.6	807.8	43.6	Moderate	No	CGCTTTGCGGCCGCCTAATGATGATGATGATGATGTGACTCATCTGTCTGTGGAATGTTG
ASP2NOTHIS	27629-002	56	DST	0.20	17271.2	82.8	32.4	22.8	714.5	41.4	Moderate	No	CGCTTTGCGGCCGCTCAATGATGATGATGATGATGCTTCAGCAGGGAGATGTCATC

PCR 100ng 1μl of Asp2pcDNA3.1hygro+ ®
32μl H_2O
8μl dNTPs
5μl 10x buffer
1.5μl Asp2 Bam
1.5μl Asp2not-tmHis or Asp2not His
1μl Pwo I

x 2 — 15 cycles 9700

Extract: ppt rxns. Resuspend ± tm in 4μl H_2O
5μl 10X #3
2μl Bam
2μl Not

37°C o/n

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[signature]

His Tagged
Asp2-tm Bam-Not
Asp2 Bam-Not

Run 1% agarose prep gel:

Cut out fragments & geneclean

Conc by OD =
Asp2-tm His = 80ng/λ
Asp2+tm His = 60ng/λ

Ligations : 120ng pVL1393/Not Bam = 3μl
Asp2 insert 2μl
10x buffer 1
T4 ligase 1
H_2O 3

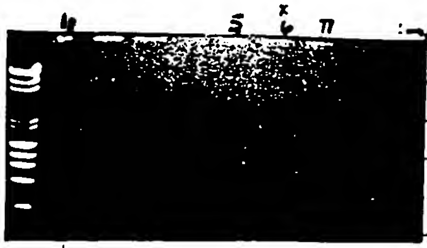
16°C o/N

TF DH5α w/ 2μl. Plate 200μl on LB+Amp. Inc @ 37°C o/N

Good # of CFUs over background - Pick 7 CFU / construct & PCR w/ Asp2 (2-5) to yield @ ~ 440bp frag.)

Nice Neg Control for the -tm's - the primer (Asp2-2) is in the TM - take a guess & pick #6 for each construct
Inoc 1 liter LB+Amp-soln. @ 37°C o/N

Asp2 - tm His
PCR'd : 2-70-Bam & 2-70-Sph



: Work up std alkaline lysis preps.
Band on Cs - also re PCR -tm bugs w/ new set of primers (2-70-Bam & 2-70-Sph)
There are weak bands in 1, 5, 7 - I don't see anything in #6 but I'm hoping that is due to not having much to amplify in the PCR after using most of the 50λ for the inoculation.

Harvest nice plasmid bands, extract & dialyze/H_2O o/N

Read and understood by me

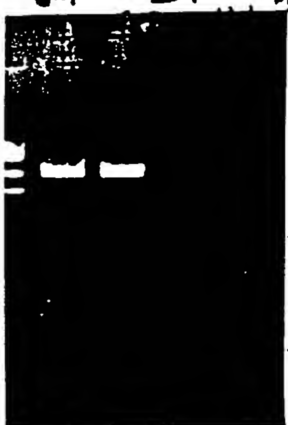
Date

Ppt & Resuspend in 1ml H_2O

Asp2 + tm His pVL1393 = 1.9 μg/μl
Asp2 − tm His pVL1393 = 2.3 μg/μl

Test digest 1μl w/ 4μl Bam + 4μl Not in 100μl 1X #3 for ~4 hrs @ 37°C

Asp2± tm His pVL1393



Look good. Submit to DSC

+TM = A (12r, 13f, 14f, 15r, 16f, 29f, 30r)
−tm = B (")

Note to BEVS to begin baculo expression
Samples given to Ma Li to transfer to Merrill Babcock

Rec'd seq. from Roger @ /DSC930-934

The Seq's are correct!

Bam-Not sites intact
6XHis fused on C-terminal followed by STOP
Alignment w/ 2R Seq − 100% identity except ambiguity that has always existed between 2R & SKB — Silent w/ no AA change

Read and understood by me

Date

Pro Asp2 pQE70 Analysis

See 31942 p. 145-148 for Construction, etc.

4 CFUs from the Asp2pQE70 mp #6 / M15(pREP) transformation were picked & grown & induced w/ 1mM IPTG.
The induction was not obvious.

Next actions: Digest mp #6 looked ok ∴ Send up for sequencing
Rerun more sample T_0/T_4 on a 10 well gel.

Reran 10µl of T_0/T_4 samples after mechanically shearing w/ a 22g needle, followed by reboiling 5'. The induced time points pinched & smeared while the T_0's looked ok.
Induction of a single band isn't obvious —
The #6 mp DNA was submitted for Seq w/
12r, 13f, 14f, 15r, 16f, pQE30r labeled Pro70
— pQE70 forward (pQE promoter) primer was ordered.

Obtained preliminary Seq from Roger. There are 2 bp deleted & a substitution relative to the correct Seq.

Roger "found" the 2bp deletion — but the 1st substitution which changes N → T can't be read from either strand ∴ resubmit w/ primer #16f & give Roger 2x11µl DNA so he can add some of his own Asp2 primers

Jerry said the samples were dropped — resubmitted this A.M.

Even if there is an amino acid substitution, I should be making & inducing a protein since all is in frame w/out STOPs.

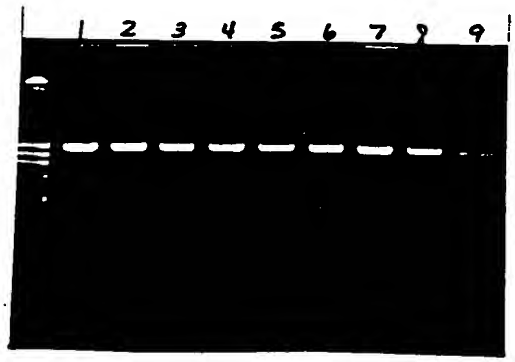
Roger left a message saying "It's an A" which makes the Seq correct

Read and understood by m

Date

Pick 9 CFU from Asp2 $^{\#6}$pQE70[M15pREP] & PCR to verify inserts w/ 2-70-Spn & 2-70-Bam for 35 cycles:

Asp2pQE70[M15pREP] 2-70-Spn & 2-70-Bam



All are positive as would be expected since the M15's were tf w/ supercoiled mp #6 DNA.

Inoc 2x5ml LB + 100µg/ml Amp + 25µg/ml Kan w/ #1-4 - Grow 2½ hrs - freeze 1x 5ml aliquot

IPTG induce the other. (1mM) for 3 hrs - freeze culture

Thaw cultures ck OD of 1ml (* = IPTG induced)

1	0.464	pellet 1ml ~ 0.500	resuspend in 50µl Et/SDS Δ100°C 5' Add 15µl H_2O 25µl 4X NuPAGE S.B. 10µl reducing agent
2	0.554		
3	0.475		
4	0.428		
*1	0.823	pellet 0.5ml ~ 0.5 OD	
*2	0.895		
*3	0.928		
*4	0.921		

Run 2 NuPAGE 10% MES gels w/ 10µl of each sample
marker 1 1*, 2 2* etc double marker.

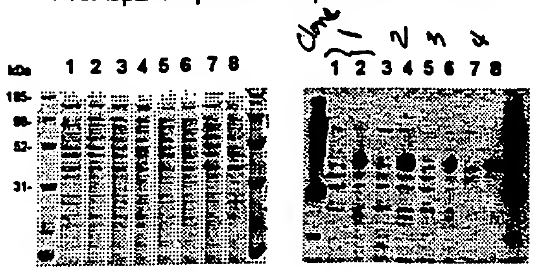
Stain 1 gel in collodial blue
Western blot 1 gel - probe w/ Pierce INDIA His-Probe Super Signal

Stain shows no obvious induction, but the His-probe clearly picks up an induced band in all 4 clones.

A very faint band can be seen that corresponds

[signature]

ProAsp2-TMpQE70 Expression in E.coli



Coomassie stained

Ni²⁺Activated HRP probed

Inoc 400ml LB + Amp + Kan w/ #1. Grow @ 37°C o/N

Grow 4 liters LB + Amp + Kan
Inoc each w/ 100ml o/N culture. Grow 2½ hrs
Induce w/ IPTG to 1mM for 3 hrs

Spin down bugs & transfer to M. Fairbanks

Mike says he sees the His signal in the <u>Soluble</u> fraction — but at very <u>low levels</u>
Will try a time course to try & boost expression

In an effort to boost expression — Try a <u>time course</u> & <u>Switch to Clone #2</u>

5ml LB + 25µg/ml Kan + 100µg/ml Amp

Inoc 8×5ml LB + <u>200µg/ml</u> AMP + 25µg/ml Kan w/ 50µl o/N
Grow @ 37°C 2½ hrs

Induce 4×5ml w/ 1mM IPTG
4×5ml w/ 2mM IPTG

Collect time points @ 1hr, 2hr, 4hr, o/N → Store on ice @ 4°C

ck OD A_{600} of each culture. Pellet 1 OD of each & give to M. Fairbanks for analysis

M. Fairbanks reports <u>no</u> expression.

Now Heinrickson reports that Jordan Tang's Collegues

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Date

have successfully expressed active Asp 2 in E coli
Suddenly the focus has shifted back to E coli expression

Trying to work w/ the ProΔTM pQE70 construct we have in hand & optimize expression:
TF ProAsp2pQE70#6 mp DNA into XL-1 Blue MRF'Kan
DH5α F'IQ
Plate on LB+Amp (100μg/ml) + Kan (25μg/ml) plates incubate @ 37°C O/N

Prepare:	LB-LS	LB-HS	2XYT	SuperBroth
Tryptone	10	10	16	35
Yeast Ext	5	5	10	20
NaCl	5	10	5	5

Pick 2 CFUs from each transformation → 50μl H_2O
Aliquot 10μl to 10ml of each broth. incubate @ 37°C w/ Shaking. After 8 hrs @ 37°C ck ODs

GAM

Bug	Clone	broth	OD/ml	OD/ml induced
XL1 Blue	1	LB-LS	0.325	1.16
"	1	LB-HS	0.511	1.75 remove 0.5 OD worth &
"	2	LB-LS	0.274	1.73 pellet in 2059 tube.
"	2	LB-HS	0.391	1.74 Store @ 4°C O/N
DH5α	1	LB-LS	0.345	1.67
"	1	HS	0.391	1.69 Induce 5ml w/
"	2	LS	0.132	1.68 50μl 100mM IPTG
"	2	HS	0.103	1.70 (1mM) @ 30°C
XL1 Blue	1	2XYT	0.543	1.92
"	2	2XYT	0.430	1.92 O/N (14½ hrs)
DH5α	1	2XYT	0.483	1.89
"	2	2XYT	0.147	1.9 Remove 0.5 OD of
XL1 Blue	1	Super	0.617	1.99 Induced & pellet
"	2	"	0.416	1.97
DH5α	1	"	0.413	1.99
"	2	"	0.116	1.94

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Date: Sep 69

E.Coli Expression: Asp2 Pro Form

Ordered the oligos to allow expression of the Pro form minus the TM in E coli using the Qiagen vector pQE70

The 5' oligo will incorporate an ATG embedded in an SphI site, then start with the amino acid sequence QHGIRL...

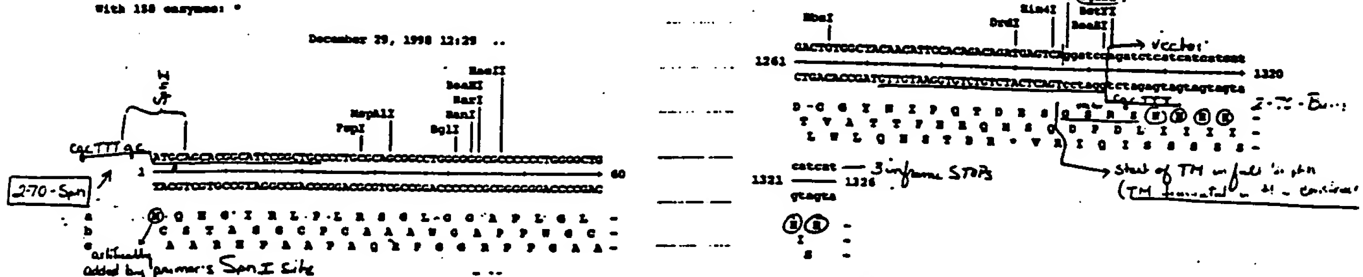
The 3' oligo will add a BamHI site immediately 3' to the last amino acid 5' to the TM. The 6 His tag will be incorporated on the C-terminus by the vector.

(Linear) (mapsite=) MAP of: asp2pqe70.seq check: 6901 from: 1 to: 1326

the proform of asp2 truncated at the transmembrane with a 5' sphI and a 3' BamHI site in the Qiagen vector pQE70

With 158 enzymes: *

December 29, 1998 12:29 ..



Oligo Name	Oligo #	LEN	Pur	Scale	MW	Tm	µg/OD	OD	µg	nmol	2ndary	Dimer	Sequence (5'-3')
GEL STANDARD	10mer, +6mer												
2-70-SPH	25824-001	30	DST	0.20	9131.0	89.2	34.5	48.8	1681.1	184.1	Strong	No	CGCTTTGCATGCAGCACGGCATCCGGCTGC
2-70-BAM	25824-002	37	DST	0.20	11332.4	81.0	33.6	49.3	1658.0	146.3	Weak	No	CGCTTTGGATCCTGACTCATCTGTCTGTGGAATGTTG

PCR Asp2 pcDNA3.1hygro & Asp2S pcDNA3.1hygro

1µl - 100ng template
5µl 10x Pwo buffer
1.5µl Sph primer
1.5µl BamHI primer
8µl dNTPs
32µl H_2O
1µl Pwo I

} 2 rxns — 2, 2S — 15 cycles

Ppt, pellet resuspend in 82µl H_2O, 10µl 10x #2, 4µl Bam, 4µl Sph } 37°C O/N

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Also 3µg pQE70 in 4µl

SW

PLEASE LEAVE RED SPACES FOR MICROFILMING

Run 1% prep gel – see p. 149

Geneclean pQE70/Spn-Bam
Asp2/Spn-Bam } Resuspend in 50µl

Toss Asp2S – the seq has 2 nt deletions per J. Slightom

I accidently loaded these fragments back on a gel – cut out & hold @ 4°C

Geneclean frags }
ck conc of frags by OD
Asp2/Spn-Bam = 35ng/µl
pQE70/Spn-Bam = 15ng/µl

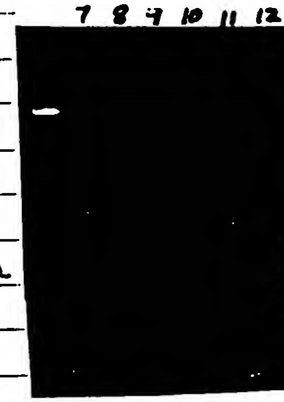
Ligation:	③	④	
	70ng Asp2 = 2µl	—	16°C o/N
	90ng pQE70 = 6	6	
	10X buffer 1	1	
	Ligase 1	1	
	H_2O —	2	

Note
TF DH5 w/2µl because of the higher tf efficiency – then
Retransform later into (M15pREPs)
Plate 200µl on LB-Amp Inc @ r.t for the weekend

Only 6 CFU – Pick & PCR w/ Spn-Bam PCR primers.

No Inserts!
Start Again w/ the PCR 100ng Asp2pcDNA3.1 = 1µl
dNTPs 8
10X buffer 5 x2
2-70-Spn 1.5
2-70-Bam 1.5 15cycles
Pwo
H_2O 32

Pro Asp2pQE70
2-70-Spn-2-70-Bam
7 8 9 10 11 12



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Extract ē ppt rxns. Resuspend in 41µl H_2O, 5µl 10X #2, 2µl Spn, 2µl Bam

Also Digest more pQE70 : 5µg = 10µl

10X #2	5
Bam	2
Spn	2
H_2O	31

Asp2 Pro Spn-Bam | pQE70 Spn-Bam

Inc @ 37°C o/N

Gel purify w/ geneclean

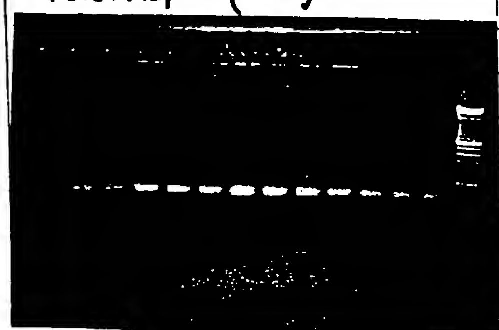
Conc by OD = pQE70/Spn-Bam = 40ng/λ
proAsp2/ " = 20ng/λ

Ligations: 2µl pQE70 = 80ng
1µl 10X
1µl ligase
6µl proAsp2

Tf DH5 w/ 2µl. Plate 200µl on LB+AMP

High background. Pick 14 CFUs ē PCR w/ Asp2 (2-5) to produce @ ~440bp product.

PCR: Asp2 (2-5)



Nice Neg Control! the primer pair I chose for PCR included the TM ∴ these CFUs may not be neg. Repeat using 2-70-Spn ē 2-70-Bam

PCR - 2-70-Bam
2-70-Spn

ProAsp2 pQE70

ProAsp

Faint bands in #6 ē #7
Inoc 5ml o/N w/ #6, #7 for mini preps

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JW

Work up std. alkaline lysis mini preps from 1½ mls. Resuspend in 100µl H_2O

Test digest 5µl w/
6µl 10X #2
2µl SpnI
2µl BamHI
45µl H_2O
} 2 hrs @ 37°C

P10 Asp Z pRE70 mp

#6 looks ok albeit light. Tf M15(pREP) 200µl w/ ~0.5µl of #6 & #7. Plate 50µl on LB + Amp + 25µg/ml Kan. Inc @ 37°C O/N

Pick 4 CFU & inoc 5ml LB + Amp + Kan O/N
Inoc 10ml LB + Amp + Kan w/ 500µl of each O/N
Grow ~2 hrs to O.D 0.5 – 0.7

T_0 1	0.555 OD/ml	pellet 1 OD – Store @ –20°C
2	0.57	
3	0.741	
4	0.653	

Add IPTG to 1mM & Continue Growth @ 37°C. Measure OD @ 2hrs

T_2 1.	1.118	pellet 1 OD Store –20°C	T_4 1.	1.386	pellet 1 OD & Store @ –20°C
2	1.144		2.	1.434	
3	1.267		3.	1.464	
4	1.177		4.	1.275	

Resuspend 1 OD equiv's in 65µl Et/SDS. Δ 100°C 2". Add 25µl Novex 4X S.B & 10µl reducing agent Δ 70°C 10'
Run a 15 well NuPAGE gradient gel – loading the 5µl was very difficult due to stringy viscosity (DNA?) Stain gel in Collodial blue
Nothing jumps right out & the loads seem light.

For further analysis See 32587 p. 31

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Date

SW

Transfection of Sf9 cells with Asp2ΔTM
(from Bienkoski's lab)

(1) Use 2×10^6 Sf9 cells for transfection.

(2) Add 0.5 μg of virus DNA and 2 μg of transfer DNA.

(3) Incubate at 27°C for 4 hr.

(4) Add 4 ml of TNM medium and keep at 27°C for 5 more days.

______ . I did co-transfection today, and the cells were incubate at 27°C for 5 more days.

______ . Transfection stock was harvested and labeled this morning and it was stored at 4°C.

______ . I did plaque assay today with 6 dilutions of the transfection stock. The plates were kept at 27°C for 6 to 10 more days.

______ . Five clones were picked up and 5 ml of TNM medium was added into each clone. They will culture for 3 days at 27°C.

______ . The 1st Amp stock was harvested and labeled this morning. It was stored at 4°C.

______ . I did 2nd Amp today and it were kept at 27°C

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for 64 hr.

Harvested all 5 clones this morning. They were labeled as 2nd Amp stocks and stored at 4°c. Mike came over to picked up both pellets & sups of all 5 clones for assay.

Mike sent me a note said, there is no expression in all 5 clones. He asked to repeat the small infection in the serum free medium, he will assay them again.

I asked Jerome to repeat the small infection of all 5 clones in the serum free medium for Mike.

Jerome told me that after small infection in the serum free medium, Mike chose clone #1 for making a 100 ml of prep.

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Expression Analysis of BVES-Hu Asp2L ΔTM

Purpose: I previously analyzed the analytical scale sf9 cell infections w/ pVL1393/Hu Asp2LΔTM and concluded that (1) Clone # 1 was best for scale-up & (2) it appears that little if any of the target protein is secreted into the medium.

Experimental:

(1) More detailed analysis of the Conditioned Medium

Inspection of the WB's of the concentrated conditioned medium did reveal a weakly stained band @ ~65kDa that did not appear in the sf9 control (although this may have been due to variable serum content). Since the large albumin band might obscure any secreted Asp2L ΔTM, ~~I decided to fractionate~~ and the Asp2LΔTM content of the medium may be too low to readily detect by WB analysis, I decided to fractionate the CM.

The protein content of the conditioned medium was quantified using the BioRad method and the results are summarized Below.

CM Sample	A^{595} (25 μl)	μg/μl	Total (mg)
sf9 control	0.135	0.18	8.1
AcNPV-CDK5-3	0.132	0.17	7.3
Hu_Asp2LΔTM	0.119	0.16	7.2

20 ml aliquots of AcNPV-CDK5-3 and Hu Asp2LΔTM conditioned medium were dialyzed against 4 l (2x) 25 mM NaOAc (4.5) O/N @ 4°C. This resulted in some ppt so the solutions were clarified by centrifugation (3000 rpm / 15') & the protein assay repeated.

Sample	Suspension	Super
AcNPV-CDK5-3	0.119 / 0.16 μg/μl / 3.5 mg total	0.041 / 0.06 μg/μl / 1.3 mg
Hu_Asp2LΔTM	0.092 / 0.13 μg/μl / 2.8 mg total	0.039 / 0.06 μg/μl / 1.3 mg

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Based on the protein assay, the recovery following dialysis is as follows:

AcNPV-CDK5-3 1.3mg/3.5mg x100 = 37%

Hu Asp2LΔTM 1.3mg/3.2mg x100 = 40%

The clarified supernatants were chromatographed on a Mono S column equilibrated in 25mM NaOAc (4.5) as follows

FR = 1.0ml/min
Sample Load ≈ 22mls
Elution 0 → 100% B, 50' where A = 25mM NaOAc (4.5)
B = " /1.0M NaCl

The elution profile was monitored @ 280nm (0.05AUFS) & 1.0ml fractions were collected for further analysis.

16.25μl samples were taken for NuPAGE gel analysis as usual;

→ 1X loading buffer + DTT + sample $\xrightarrow[10']{70°C}$ $\xrightarrow{10\lambda\ load}$ 4-12% gradient gels (15well)

→ 1X MES R.B. / ET, 90' @ 35V

→ WB — 1/1000 dil UP191TB#4
— 1/2500 dil G αR-AP
— NBT/BCIP

A second gel/WB was run (based on the first gels) to reanalyze the following samples on a 10well/10% gel

1. CHO Asp2L #5 (20)
2. CM-BVES Asp2LΔTM
3. " " " pH4.5
4. Mono S #10
5. Mono S #12
6. " #14
7. " #16
8. CM (cdk5) cnt
9. CM (cdk5) control pH4.5)

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Results:

Aliquots of conditioned medium obtained from sf9 cell infections w/ recombinant bv containing Hu Asp2L ΔTM or a control gene (Cdk5-3) were analyzed for the presence of Asp2ΔTM protein, before & after chromatography on Mono S. Dialysis of the conditioned medium against NaOAc buffer pH4.5 led to protein ppt of ~2/3 of the original protein. The supernatant was fractionated by Mono S chromatography & the elution profiles monitored by $A^{280\,nm}$ absorbance & WB analysis. Both CM samples showed similar behavior, summarized below

* ~2/3 of starting protein ppt upon dialysis
* large A^{280} in unbound fraction of Mono S
* similar A^{280} fingerprints during the gradient elution.

To determine which fractions contained Asp2, aliquots were analyzed by WB analysis & the results are summarized below:

* Conditioned Medium ~45kDa immunoreactive band
* Dialyzed CM (Super) ~43kD " "
* PPT from Dialysis — blank
* Mono S column fractions — maybe immunoreactivity in #12

Band (immunoreactive) size decreased following dialysis — ? Activation ??

2ND Analysis

* BVES-CM Asp2LΔTM -> immunoreactive band @ 52kDa that is not in the control
* BVES-CM Asp2LΔTM/pH4.5 -> immunoreactive band @ ~50kDa, but much lighter than before (not in control)
* faint immunoreactive band in #12/14

* Seems like Asp2ΔTM is going away w/ time (1) loss in SM + (2) lack of conc in col

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Continued Analysis of BVES-Hu Asp2L ΔTM Conditioned Medium

Purpose: To determine if purification of the proform by anion exchange chromatography followed by activation @ low pH would be a sensible strategy.

Experimental

The starting material for this work was described on pp 110-112 (both Hu Asp2L ΔTM and cdk5 control medium). Twenty ml aliquots of each CM sample were dialyzed against 4L of 25mM Tris-HCl (pH 8.0) O/N @ 4°C. A slight amount of ppt was noted following dialysis so the protein content of the dialysate was quantified before & after centrifugation (3000 rpm, 15')

Sample	A^{595nm}/25µl	µg/µl	total µg	Δ
BVES-Asp2L ΔTM CM-dialysate	0.136	0.18	4,050	
" " -super	0.121	0.16	3,600	450µg
BVES-cdk5 CM-dialysate	0.122	0.16	4,000	
" " -super	0.118	0.15	3750	250µg

The clarified supernatant obtained following dialysis/centrifugation was chromatographed on a MonoQ column under the following conditions:

- Load ~22ml @ 1.0ml/min w/ 25mM TRIS-HCl (8.0)
- Wash w/ 25mM TRIS-HCl (8.0) until A^{280} reduced (to ~30%, never reached 0)
- Elute w/ a 30' gradient from 0→1.0M NaCl in 25mM TRIS-HCl (pH 8.0)
- Elution profile monitored by A^{280nm} (0.1 AUFS) & WB of individual fractions.

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(ii) Gel/WB Analysis:

Aliquots of various fractions were taken for NuPAGE gel separation & WB analysis. Due to a fraction collector mishap, I lost a good portion of the column fractions between #1 → ~17. There was enough to analyze by gels. Also, in the case of the pH 8.0 ppt, the material (~0.45 mg protein by difference) was resuspended in 0.45 ml dH_2O.

Lane	Sample - gel 1	Sample - gel 2
1	CMBVES-Hu6p21LOTM	8
2	dialyzed CMs (pH8)	9
3	ppt from dialysis	10
4	V_o I	11
5	V_o II	12
6	4	13
7	5	14
8	6	17
9	7	19
10	STD	STDs

16.25 µl sample + 6.25 µl 4x LB + 2.5 µl DTT
↓ 70°C, 10'

Following electrophoresis (200V, ~45'), the gel was electroblotted to PDVF (35V, 90') & immunoreactive material visualized using UP-191-TB#4 as usual (1/1000 dil UP191-TB-#4 / 1/2500 GαR-AP)

(iii) Low pH Treatment of Fraction 11

The protein content of #10, #11 & #12 was determined using the BioRad Assay. 8 µl of each fraction was run on a 10% NuPAGE gel & visualized by silver staining.

#10	0.22 µg/µl	× ~60 µl	⇒ ~13 µg Total	(220 µg)	~940 µg
#11	0.29 µg/µl	× ~50 µl	⇒ ~14 µg "	(290 µg)	
#12	0.43 µg/µl	× ~60 µl	⇒ 26 µg "	(430 µg)	

16 µl of #11 was mixed w/ 1.6 µl 1.0 M NaOAc (4.5) and incubated o/N @ 4°C. This material was then run in duplicate on a 10% NuPAGE gel as usual (reducing) & ½ of the gel stained w/ silver & ½ transferred & stained w/ Ab.

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Results:

I previously showed a shift in the Mr of immunoreactive Asp2LΔTM derived from the Conditioned medium of BVES infection upon acidification to pH 4.5. Since this material appeared unstable (immunoreactive band ↓ w/time) I reasoned that it might be better to partially purify the pro-form & activate under the conditions. For this reason, an aliquot of the BVES CM from Asp2LΔTM was exchanged w/ 25 mM TRIS-HCl (pH 8.0), chromatographed on MONO Q & the elution profile monitored by A^{280} absorbance & WB analysis.

Dialysis of the CM caused minor ppt of protein (~10%) & the clarified super was fractionated by anion exchange chromatography. A considerable amount of material did not bind to the column & there was no detectable immunoreactive material in the Vo. Gradient elution with a steep NaCl gradient (0→1.0M, 30') resolved multiple A^{280} peaks that eluted between 0→0.5M NaCl. WB analysis of these fractions revealed a strong concentration of immunoreactive material of the expected Mr in fractions 11 >> 10/12, well separated from the bulk of the A^{280} absorbing impurities. (Note that a shallower gradient might improve the resolution). This immunoreactive material corresponded w/ an A^{280nm} peak eluting @ ~ 0.3M NaCl.

Gel analysis of the immunoreactive fractions & silver staining revealed a relatively simple pattern of polypeptides & it was clear from comparison with the immunoblot of the same fractions that which band corresponded (intensity & position (Mr)). In an attempt to reproduce the observation of 'activation' in the acidified of the CM, fraction #11 (an aliquot) was incubated @ pH 4.5 o/n @ 4°C & the products visualized by both silver stain & WB. The silver stained gel showed a smear, rather than a discrete band, in both #11 & #11, pH 4.5 & a number of additional changes. Alternatively, the Western blot showed a discrete reduction in the observed Mr of the pH 4.5 treated sample, consistent w/ removal of the NH_2-terminal prosegment.

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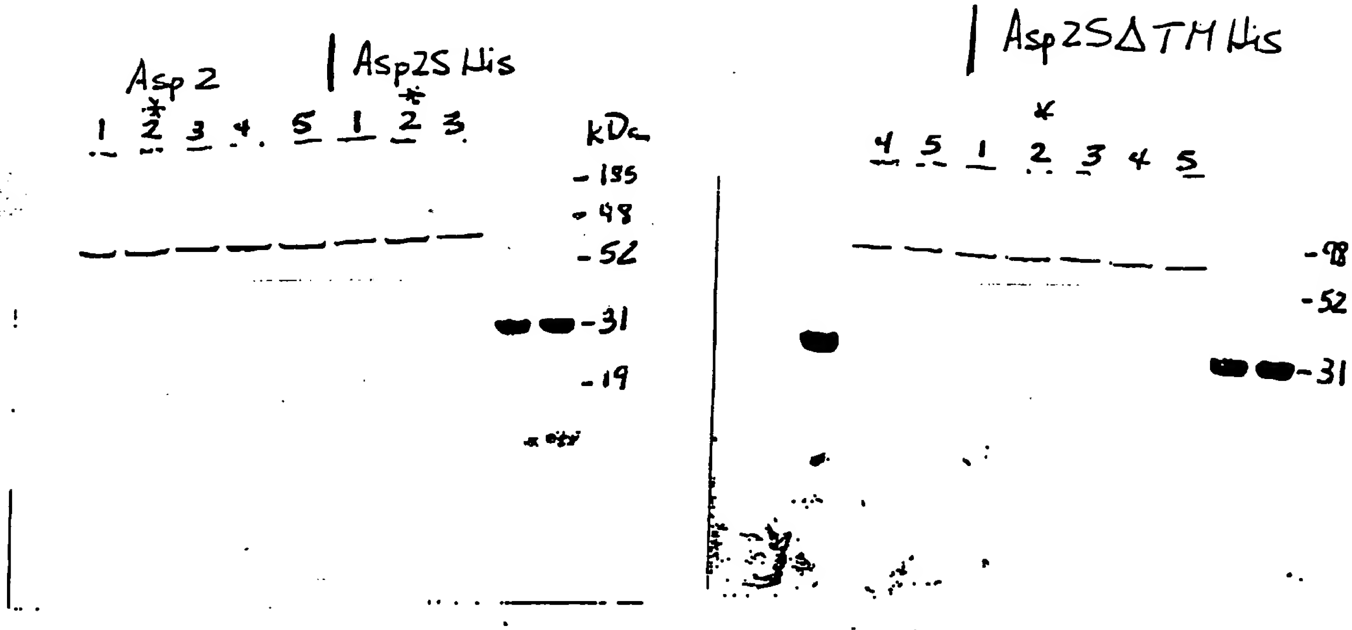
AP Western blots of Baculovirus clones
Probed UP-191 (Asp2) TB#4@1:1000



#2's → Scale up

TF High Fives w/ Asp2 ΔTM & Asp2 ΔTM His / pIZ

These Constructs were made by cutting pVL1393 ΔTM & ΔTM His w/ Bam + Not (See p. 87-90) & inserting into pIZ/V5-HIS not using vector V5 or His

High fives (H5) have been in culture in SF High Five media + Gentamycin for 6 passages & are behaving nicely

Dislodge cells into media, pipet vigorously & count.
Seed ~2×10^6 cells / 60mm dish

- Plate 1 dish for each of 3 transient time points (24, 48 hr, 5 day) and 2 for Stables // Construct plus liposome only

- Rock gently for ~3 mins. Let cells attach for ~20 min
- Prepare TF reagent: 1 ml SF media
  5 µl 10 µg DNA ATM & ΔTM His } for each
  20 µl Insectin plus } 60mm plate
  Vortex 10 sec, Set at r.t ~ 15 mins.
- Remove media from plates — Add DNA/liposomes dropwise.
  Rock @ r.t (2/min) for 4½ hrs
- Add 2ml SF media → Inc w/ wet paper towels in Sealed bag

- Harvest 24 hr time points — pipet cells into media to loosen. Spin 1.5K 5 mins to pellet the cells. Harvest the Culture media & cells separately. Store @ -20°C

- Harvest 48hr time points as above.
- Add Zeocin selection to stables: remove media from 2×60mm dishes for each Construct. Resuspend in 10 ml SF media no Antibiotic. Transfer to 150 mm dishes. Allow the cells to sit down ~30 min r.t. Remove media & replace w/ SF media + 400µg/ml Zeocin

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[redacted] [redacted]

- Harvest 4½ day (108 hr) time points
  Run a 4-12% NuPAGE w/ 26 μl media (from 3 times / 2 constructs)
  10 μl 4X NuPAGE SB
  4 μl reductant
  Stain w/ colloidal blue → ~~no~~ bands in media only control
  (the conditioned media from all samples show a ladder of bands - one of the darkest bands in the samples ē one that seems to intensify with time is running @ 52 kDa - Western is in order

- Refeed Selection plates — mass killing evident

Run sample gel exactly as above. Electroblot 1 hr. Probe PVDF membrane w/ UP191 (Asp2) @ 1:1000. Develop via AP.
The 52 kDa band is positive!!! Silver stain of the transferred gel shows uneven transfer ∴ The His tagged versions seem to be expressing very well too!
108 hrs is the best of the 3 timepoints.

Refeed Zeocin selection plates
Refeed Zeocin " → pockets of adherant cells evident
HJB refed once while I was gone. There are thousands of sparsely placed single cells attached all over the plates — no foci evident. (Val Ruff OU7252 is doing a stable transfection in parallel ē sees the same thing — even on her liposome (no DNA) plates)
∴ I think we may not have achieved complete killing of non resistant cells ∴ Split 1:2 → 400 μg/ml Zeo
→ 600 μg/ml Zeo

Floating cells in both 400 ē 600 cultures refeed w/ respective media

On advice from Invitrogen tech. rep. remove selection ē allow foci to form.

Add 25 ml media w/o antibiotic to selection dishes

Toss dishes

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Large Scale Transient High Five Transfection

Plate 5 x 100mm dishes for each construct: p1Z Asp2ΔTM
p1Z Asp2ΔTMHIS

Cells: 6 x 10^6/dish
media: 3ml SF media + gentamycin
DNA: 30µg (ΔTM & ΔTM HIS)
InsectinPlus: 60µl

Plate cells, rock 3 min. Let cells attach for ~20mins
Combine media + DNA + liposomes. Vortex. Inc @ r.t 15min
Add dropwise to plates. Rock 2 rpms 4 hrs

Add 6ml SF media. Store @ r.t on wet paper towels. MJB to harvest @ 4½ days.

Mike & Monica report tons of protein is being expressed & secreted into the media.

2nd Large Scale Transient

(~4 x 10^7 cells/confluent T150)

Scale up to 150mm dishes X 20
cells: 1.2 x 10^7
media: 12ml SF media + gentamycin (6ml for transfection)
DNA: 60µg (Asp2ΔTM HIS)
liposomes: 120µl InsectinPlus

Still dividing & happy

⇒ Put 25µg/ml Zeo on one of the 150mm dishes (to select Stables)
Refeed w/ 250µg/ml Zeo
Harvest ~250ml transient conditioned media → Monica for purification

Monica reports β-secretase substrate activity –

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